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# RIBONUCLEIC ACID AS A TRANSFORMING PRINCIPLE IN BACTERIA* **

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Since the discovery of the capsule transformation of *pneumococcus* by Griffith[1] and the finding that the purified transforming principle has all the properties of a highly polymerized deoxyribonucleic acid (DNA) by Avery, MacLeod and McCarty[2], many examples of bacterial transformations have been reported and evidences have been given that the transforming principle concerned is exclusively DNA. The work of Avery *et al.* has been generally regarded as a great contribution to modern biology, and the transforming principle DNA is believed to be equivalent to the bacterial gene for the geneticist.

In studying the biological function of the nucleic acids with the special reference to the transmission of hereditary information, we made use of transforming principle of penicillin-resistance of *Bacillus subtilis*. The results of our experiments showed that the chemical nature of this transforming principle could not be attributed to DNA as generally recognized. It was identified as RNA. The discovery of this transforming RNA is especially interesting to the biochemistry of genetics, since so far no experimental evidence has ever been presented to demonstrate RNA as a primary genetic material as DNA is in the cellular form of living systems.

## MATERIALS AND METHODS

The penicillin-resistant strain of *B. subtilis* IRC-7[1)] was obtained by training the bacteria to grow continuously in a medium containing 100 $\mu$g/ml of penicillin G. The crude transforming principle was prepared by the desoxycholate method of Avery *et al.*[2]. Isolation and purification of RNA

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1) *B. subtilis* IRC-2 and IRC-7 were kindly given by Prof. A. Braunstein of the Institute of Medical and Biological Chemistry, Academy of Medical Sciences USSR.

were carried out according to Kirby[3] with some modifications. A penicillin-sensitive strain *B. subtilis* IRC-2 was employed as the recipient bacteria to the transforming principle. It produced little inducibility or mutability in the experimental conditions. The transformation experiment was performed essentially as described elsewhere[4]. Competent bacteria of the penicillin-sensitive strain IRC-2 were treated with the transforming principle in a specific medium which contains 0.1 M glycerol, 0.01 M $(NH_4)_2SO_4$, 0.1 M $KH_2PO_4$, 0.1 M $Na_2HPO_4$, 0.01 M NaCl and 0.001 M $MgSO_4$. The exposure of bacterial cells to transforming principle was terminated by adding DNase or RNase at the given period. For assaying the transformants, two procedures were run in parallel: (i) The exposed bacteria were transferred to 20 ml of the ordinary nutrient medium which contained 100 $\mu$g of penicillin per ml and was shaken at 37°C. After the appearance of the transformants, the number of bacterial cells was estimated turbidimetrically. (ii) The treated bacteria were transferred to penicillin agar plates incubated at 37°C. The number of colonies was counted. In both instances sufficient time was allowed for phenotypic expression. The first method was more rapid in estimating the transforming activity, but it usually gave much higher number of transformants, because divisions of the transformed cells could not be avoided. For quantitative measurement of the activity of the transforming principle, the plating method was preferred. Here the number of colonies appeared actually represented the number of transformed bacteria.

The activity of penicillinase was assayed by the conventional manometric method[5].

Crystalline pancreatic Ribonuclease (RNase) and Desoxyribonuclease (DNase) were manufactured by L. Light and Co., England. They were checked against thymus DNA and yeast RNA. The RNase was found to depolymerize the latter only without touching the former, while the DNase acted the other way round. Further, a mixture of RNA and DNA such as the crude transforming principle, after progressive digestion with the RNase and precipitation, showed only decreasing proportion of RNA in the residue without any change of DNA; the opposite result was obtained with the DNase.

## Experimental Results

(1) *Activity of transforming principle.* When the penicillin-sensitive strain *B. subtilis* IRC-2 grown for about 10 hr reaching the stage of exponential growth was collected and subjected to the transforming principle treatment for 30 min, the sensitive bacteria were converted to be resistant in the penicillin medium. The bacteria which thus acquired the character of penicillin resistance retained the ability to grow in the penicillin medium even if they were maintained and transferred frequently in the medium without penicillin. Therefore the acquired character of penicillin resistance thus obtained is inheritable.

The crude transforming principle used in the experiment was a mixture of RNA and DNA. Further analysis was made on the nucleic acid component treated either with crystalline RNase or with DNase. It was shown that the activity of the transforming principle was markedly decreased after 30 min incubation with RNase and it became completely obliviated after 60 min incubation. On the contrary, the transforming activity was insensitive to the action of DNase even the time of incubation with the enzyme was prolonged to two hours (Table 1). It should be noted that the pre-incubation of recipient bacteria with RNase alone for 30 to 60 min did not influence their response to the transforming principle.

**Table 1**

Inactivation of the Transforming Principle of *B. subtilis* by RNase

| Treatment | Transformants[1]/ml |
|---|---|
| None | 0 |
| Transforming principle[2] | $1.6 \times 10^8$ |
| Transforming principle + DNase[3], 30min | $3.1 \times 10^8$ |
| Transforming principle + DNase, 60min | $1.4 \times 10^8$ |
| Transforming principle + RNase[3], 30min | $7.0 \times 10^7$ |
| Transforming principle + RNase, 60min | 0 |

1) The number of fransformants was determined by counting the bacterial cells after having grown in penicillin medium for 20 hr at 37°C with shaking, including the subsequent growth of the initial transformants.

2) The crude transforming principle contained DNA $4 \times 10^{-2}$ μg/ml, and RNA $10^{-2}$ μg/ml.

3) Crystalline DNase or RNase used in the experiments was 10 μg per ml.

(2) *Depolymerization of* RNA *and the activity of transforming principle.* To find out whether or not the highly polymerized RNA molecules were necessary for bacterial transformation in the present case, experiments were carried out to compare the degree of depolymerization of RNA or DNA with the activity of the transforming principle. The curve for the inactivation of the transforming principle versus the depolymerization of RNA was shown in Fig. 1. Incubation with 5 μg per ml of crystalline RNase for 30 min was sufficient to cause about a 40% inactivation of the transforming principle with a concomitant degradation of RNA. The exhaustive depolymerization of RNA by RNase in 60 min led to a complete inactivation of the transforming principle. On the other hand, as Fig. 2 shows, depolymerization of DNA of the transforming principle by DNase did not cause any decrease of its activity. The results of these experiments indicated that the essential component of the transforming principle of penicillin resistance in *B. subtilis* was not DNA as had been generally believed in reported cases, but a highly polymerized RNA.

(3) *Relation between the number of transformants and* RNA *concentration.* Further evidence on the chemical nature of the transforming principle was obtained from the transformation activity given by purified RNA.

Isolation and purification of RNA from the penicillin-resistant bacteria were made as follows: The bacterial lysate prepared after treatment with lysozyme in hypertonic solution was dialyzed overnight and centrifuged. To the clear

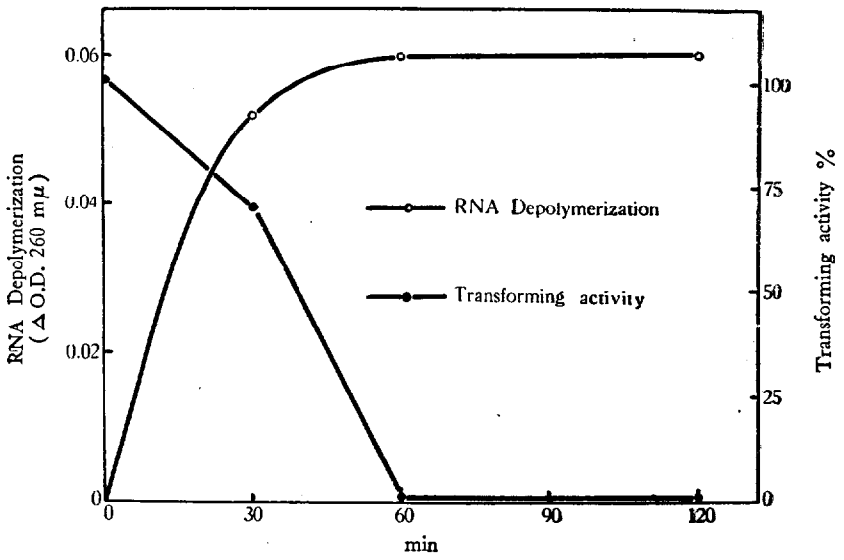


Fig. 1. Activity of the transforming principle versus depolymerization of its RNA component by RNase.

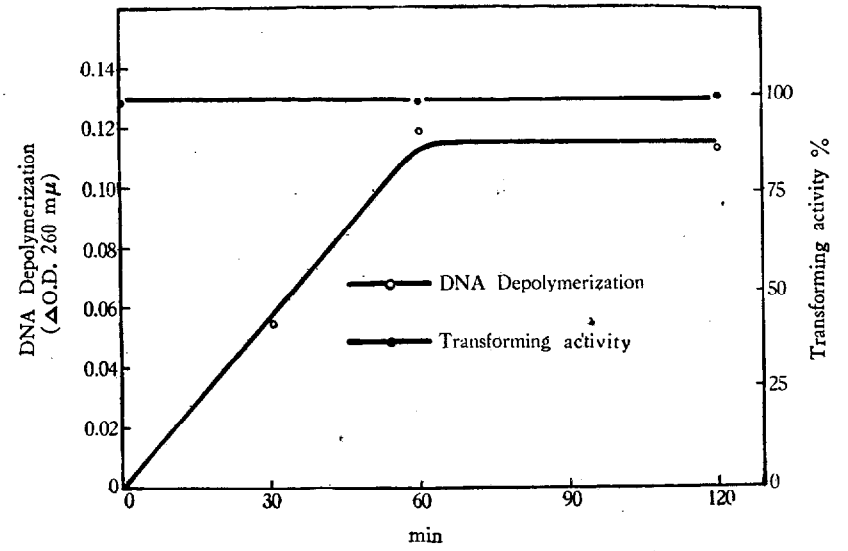


Fig. 2. Activity of the transforming principle versus depolymerization of its DNA component by DNase.

supernatant was added an equal volume of 90% phenol. The mixture was shaken continuously for 30 min at room temperature. The supernatant was separated by centrifuge and was added to an equal volume of 2% alcoholic potassium acetate. The mixture was left in the refrigerator for 30 min.

The precipitate was collected and washed several times with small amount of alcoholic solution of 0.2% potassium acetate. The precipitate thus obtained was dissolved in sterilized saline solution and was ready for experiment. It contained 7300 $\mu$g of RNA and 20 $\mu$g of DNA per ml. With such purified RNA the sensitive bacteria were readily converted to be penicillin-resistant after a short time exposure. Here again the activity of RNA was abolished by RNase.

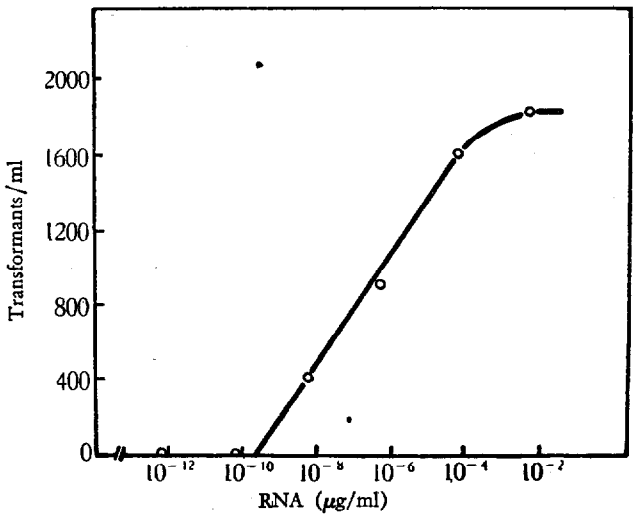


Fig. 3. Relation between the RNA concentration and the number of transformants.

For quantitative estimation of the transforming activity of RNA, the recipient bacteria ($5\times10^6$ cells/ml) were treated with different concentrations of RNA for 30 min. The treatment was terminated by RNase, and the number of transformants was assayed by the plating method. It was shown that the number of transformants increased with the concentration of transforming RNA. The maximum of transformation was reached at a RNA concentration of $8\times10^{-2}$ $\mu$g/ml. The minimal amount of RNA required to transform one bacterial cell was calculated as $2\times10^{-10}$ $\mu$g/ml. By assuming the molecular weight of RNA to be of the order of $10^6$, it can be calculated that the number of molecules of RNA necessary to transform a single bacterium in this system is of the order of $10^2$.

(4) RNA *as the carriers of genetic information.* As had been shown previously in the case of transforming DNA, the treatment of the purified RNA with trypsin or chymotrypsin so as to remove the possible contamination of proteins, did not lead to its inactivation. Both yeast RNA and the homologous RNA isolated from the sensitive strain of *B. subtilis* failed to show any transforming activity on the recipient bacteria. However, the RNA obtained from the transformants was shown to have the same activity as the original transforming RNA in imparting penicillin resistance to the sensitive bacteria. It was also sensitive to RNase.

Incubation of purified RNA with DNase, on the contrary, led to an increase of the activity (Table 2). It was due probably to the removal of the contaminated DNA which would compete with RNA on the cell surface and hinder the RNA from penetrating into the recipient bacteria.

**Table 2**

RNA as the Carrier of Genetic Information in *B. subtilis*

| Treatment | Transformants[1]/ml |
|---|---|
| Transforming RNA, $5 \times 10^{-2}$ μg/ml | $1.7 \times 10^{3}$ |
| Yeast RNA, 3.3 μg/ml | 0 |
| RNA of the Sensitive Strain *B. subtilis* IRC-7, 20 μg/ml | 0 |
| RNA of the Cells Grown from the Transformants, 35 μg/ml | $1.4 \times 10^{3}$ |
| Same + DNase | $4.8 \times 10^{3}$ |
| Same + RNase | 0 |

1) The number of transformants was determined by that of colonies on penicillin agar per ml of culture, and represented the true initial number of transformants.

The results of these experiments showed that the genetic information was carried on in the RNA molecules and indicated further that the RNA endowed with the genetic information could replicate itself.

(5) *Genetic transformation and enzyme synthesis.* It has been shown elsewhere[6,7] that the transforming principle can transmit a genetic potentiality of synthesizing certain enzymes. Penicillinase activity was demonstrated in the penicillin-resistant strain of *B. subtilis* IRC-7, whereas it was absent in the sensitive strains. When the sensitive strain *B. subtilis* IRC-2 was transformed into the resistant strain by the transforming RNA, it then possessed the ability of synthesizing the enzyme (Table 3).

**Table 3**

Formation of Penicillinase of the Penicillin-sensitive *B. subtilis* after Receiving the Transforming RNA[1]

| Strains | Penicillinase Activity (μl $CO_2$/60 min) | |
|---|---|---|
| | I | II |
| Sensitive Strain IRC-2 | 9 | 0 |
| Resistant Strain IRC-7 | 45 | 329 |
| Transformant 1[2] | 51 | 138 |
| Transformant 2[3] | 51 | 96 |

1) Bacteria grown for 15—20 hr at 37°C with shaking, the culture broth after removal of bacterial cells was used for enzyme assay.

I. Penicillinase formed in the culture not containing penicillin.

II. Penicillinase formed in the culture containing penicillin.

Reaction system: Culture broth 1.5 ml; penicillin G 10,000 units, 0.5 ml; 0.2 M phosphate buffer, pH 7.0, 0.5 ml; 0.26 M $NaHCO_3$, 0.5 ml; total volume 3.0 ml; 30°C.

2) Transformants 1: Transformants obtained immediately after exposure to RNA in selective media.

3) Transformants 2: Transformants obtained from the selective medium after several transfers on agar slants without penicillin.

In assaying of the penicillinase activity, cells from 15 to 20 hr cultures without penicillin were removed by centrifuge and the medium was employed as the enzyme source. The enzyme investigated here was exocellularly formed from the bacteria without any contact with penicillin molecules, and was therefore not an induced enzyme. The transformant culture with penicillin showed a higher penicillinase activity in the medium.

## Discussions

It is clear from the work on tobacco mosaic virus that RNA is capable of carrying primary genetic information. But no evidence for such genetic role of RNA has yet been presented in higher forms. For several decades, our interest in the genetic material has been centred upon DNA rather than any other molecules. One's idea has its root in the accumulative evidences and speculations given by various workers. It should be noted here that sometimes *a priori* conception before experimentation may lead to subjective conclusions in keeping with the traditional line and thus overlook the peculiar significance of new facts. Spizizen[7] is probably the first man who used *B. subtilis* as experimental material to study the bacterial transformation. Among the transforming principles investigated, which were all believed to be DNA, Spizizen observed a phenomenon indicating the importance of intact RNA on the activity of a transforming principle concerning sucrase formation. Unfortunately this did not arouse his attention and received no further investigation.

Our conclusion that the transforming principle of penicillin resistance of *B. subtilis* was the highly polymerilized RNA was essentially based upon the following facts: (i) The activity of the transforming principle can be destroyed by RNase but is insensitive to DNase. (ii) The purified RNA is capable of transforming the sensitive bacteria to be resistant, and the activity of the transformation is related to the concentration of RNA. (iii) The replication of the genetically donated RNA is demonstrated by the isolation of active RNA from the transformants. Although the evidence on the occurrence of the transforming RNA in bacteria is still limited, it appears likely that both DNA and RNA may serve as the genetic material according to the different conditions of the living system. It is possible that the different genetic characters could be governed by different genetic materials in the same organism, and the same character could be controlled by different genetic materials in different organisms. It would be of obvious interest to study the interrelationship between DNA and RNA on governing the genetic characters and the mechanism of their interaction. Attempts in this direction are being made in our laboratory.

## Summary

A transforming principle of penicillin resistance of *Bacillus subtilis* has been demonstrated. It is identified as a highly polymerized RNA by the

following observations: (i) The transforming principle can be destroyed by RNase, but is insensitive to DNase. (ii) There is a direct relationship between the depolymerization of RNA by RNase and the inactivation of the transforming principle. (iii) The purified RNA prepared from the penicillin-resistant strain of the bacteria possesses the activity of transformation. It is estimated that about 100 molecules of RNA are required to transform a single bacterium. (iv) The RNA liberated from the transformants is able to accomplish the transformation as the original transforming RNA which the transformants have received previously. (v) Penicillinase activity is demonstrated in the resistant strains, but is absent in the sensitive strain. The sensitive bacteria which have received the transforming RNA are also provided with the enzyme activity and become resistant to penicillin.

The discovery of this transforming RNA and its replication indicate that RNA may serve as a genetic material for certain characters in bacteria.

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